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(54) Title: TARGET GENES FOR STRAIN-SPECIFIC DIAGNOSTIC OF EHRLICHIA RUMINANTIUM AND USE THEREOF

(57) Abstract: The invention provides a combination of target genes that are useful as genetic markers for the strain-specific detection of *Ehrlichia ruminantium*. The invention also provides diagnostic methods using said combination of markers.

WO 2008/038062 A1

TARGET GENES FOR STRAIN-SPECIFIC DIAGNOSTIC OF *EHRLICHIA RUMINANTIUM* AND USE THEREOF

Rickettsiae are intracellular pathogenic bacteria responsible for various diseases on Humans and animals. *Rickettsiae* are transmitted by arthropods, most frequently ticks, lice and mites, and cause major illnesses such as epidemic typhus or Rocky Moutain spotted fever. The genus *Ehrlichia* comprises other species pathogenic for humans and mammals such as *E. chaffeensis*, responsible for Human monocytic ehrlichiosis, *E. canis*, the causing agent of canine monocytic ehrlichiosis.

Another species, *Ehrlichia ruminantium*, formerly known as *Cowdria ruminantium*, is the causing agent of heartwater or cowdriosis, an economically important disease of domestic ruminants. Heartwater can cause up to 80 % mortality in susceptible animals. *E. ruminantium* is transmitted by *Amblyomma* ticks and is present in Sub-Saharan Africa and surrounding islands, including Madagascar. Heartwater is also present in several Caribbean islands and is threatening the American mainland.

Vaccination against heartwater has long been based on "infection and treatment". Naïve animals are inoculated with blood containing virulent organisms, a procedure which carries a high risk of uncontrolled clinical reactions and the inadvertent spread of undesirable parasites and viruses. A first generation cowdriosis inactivated vaccine based on cell-cultured derived elementary bodies was developed. Although representing a considerable improvement and the first heartwater vaccine acceptable for widespread use, the level of protection conferred is still not fully satisfactory. Indeed, all animals develop a clinical reaction at challenge despite vaccination. Furthermore, livestock also faces challenge by genetically and antigenically diverse strains.

Diversity of *E. ruminantium* is a key problem which has been recognized for a long time, but insufficient information is available for optimum vaccine formulation and specific diagnostic. Serological diagnostic tests of heartwater using crude antigens from whole bacteria detect false positive reactions due to common antigenic determinants

The diversity of *E. ruminantium* was demonstrated at the antigenic level by cross-immunisation studies. Variable antigens were identified by ELISA and immunoblot using cross-absorbed immune sera.

Genetic diversity was later demonstrated when sequencing the Map 1 gene which showed a high degree of sequence heterogeneity concentrated in three hypervariable regions. Genomic polymorphism was also detected using RAPD and RFLP markers. This DNA polymorphism was shown to correlate with antigenic polymorphism.

ELISA-based and serological diagnostics have been developed using the Map 1 and the GroEL (WO 9914233) antigens. Other peptides for serological diagnostic have been described (US 2002004051, US 20020132789, WO 02/066652). Although they have dramatically improved specificity, they still display cross reaction with *E. canis* and *E.*

chaffeensis. The map1 gene initially considered as a good marker for geographic diversity, was recently shown not to be geographically constrained. Furthermore, the life span of anti-Map 1 antibodies is rather short.

PCR-based diagnostic methods represent methods of choice for the sensitive and specific detection of *Ehrlichia* in clinically reactive or asymptomatic carrier ruminants, as well as in vectors. However, in the field, hosts and vectors can be co-infested by several parasites and the diversity of pathogen species is further complicated by the existence of extensive intra-species diversity. Thus, it is important to provide means and diagnostic tools allowing not only to identify *E. ruminantium* but also to differentiate between different strains.

Sequences allowing differential diagnostic of *E. ruminantium* strain Gardel and *E. ruminantium* strain Welgevonden have been previously described by the inventors. They have shown, through complete genome sequencing and comparative genomic analysis that several genes were only found in either strain Gardel or strain Welgevonden, without counterpart in the other strain, and that several other genes, while being present in both strains differed between them by one or several mutations, such as large insertions and/or deletions that result in a frameshift and/or in a truncated version of the original gene. These genes were therefore primary targets to develop specific, multitarget diagnostic methods to differentiate between these two strains (WO 2006/045338; Frutos et al., Journal of Bacteriology.188: 2533-2542, 2006).

The inventors have now found that the use of a particular combination of some of the target genes described in WO 2006/045338 allowed not only to discriminate between strains Gardel and Welgevonden, but also in a more general way, to detect specifically *E. ruminantium* and to discriminate between a broad range of strains of *E. ruminantium* other than Gardel and Welgevonden including strains for which no genomic sequence data are available.

An object of the invention is thus the use of the following set of genes:

Erum1, defined by the sequence SEQ ID NO: 6

Erum2, defined by the sequence SEQ ID NO: 3

Erum3, defined by the sequence SEQ ID NO: 1

Erum4, defined by the sequence SEQ ID NO: 4

Erum5, defined by the sequence SEQ ID NO: 2

Erum6, defined by the sequence SEQ ID NO: 5

Erum7, defined by the sequence SEQ ID NO: 13

Erum8, defined by the sequence SEQ ID NO: 15

Erum9, defined by the sequence SEQ ID NO: 14

Erum10, defined by the sequence SEQ ID NO: 8,

as targets for the strain-specific detection of *Ehrlichia ruminantium*.

The reference sequences used herein to define the target genes Erum 1-5 and Erum 7-9 are those identified in the Gardel strain; the reference sequences used herein to define the target genes Erum6 and Erum10 are those identified in the Welgevonden strain.

However, it is to be understood that each of these genes actually exists under different allelic forms, depending on the strain of *Ehrlichia ruminantium*. The allelic forms that will be considered herein, having in view strain-specific detection, are in particular those resulting from large insertions and/or deletions that lead to a frameshift or to a truncated version of the original gene.

The invention thus provides a method for the strain-specific detection of *Ehrlichia ruminantium* wherein said method comprises determining, for each of the genes Erum 1 to Erum10 defined above, whether said gene is present in the bacteria to be tested, and under which allelic form.

Advantageously, the method of the invention is carried out by performing PCR amplification of all the target genes Erum 1 to Erum10, and checking, for each of these genes, the presence of one or more amplification product(s), and the size of said amplification product(s).

Within the target genes Erum 1 to Erum10, preferred target regions are as follows:

For Erum 1, the target region can consist of the whole sequence SEQ ID NO: 6, or of a portion thereof; in particular the target region can be defined within the portion spanning from nucleotide 1 to nucleotide 173 of SEQ ID NO: 6.

For Erum 2, the target region can consist of the whole sequence SEQ ID NO: 3, or of a portion thereof; in particular the target region can be defined within the portion spanning from nucleotide 1 to nucleotide 218 of SEQ ID NO: 3.

For Erum 3, the target region can consist of the whole sequence SEQ ID NO: 1, or of a portion thereof; in particular the target region can be defined within the portion spanning from nucleotide 1 to nucleotide 509 of SEQ ID NO: 1.

For Erum 4, the target region can consist of the whole sequence SEQ ID NO: 4, or of a portion thereof; in particular the target region can be defined within the portion spanning from nucleotide 56 to nucleotide 698 of SEQ ID NO: 4.

For Erum 5, the target region can consist of the whole sequence SEQ ID NO: 2, or of a portion thereof; in particular the target region can be defined within the portion spanning from nucleotide 1 to nucleotide 239 of SEQ ID NO: 2.

For Erum 6, the target region can consist of the whole sequence SEQ ID NO: 5, or of a portion thereof; in particular the target region can be defined within the portion spanning from nucleotide 3 to nucleotide 130 of SEQ ID NO: 5.

For Erum 7, a preferred target region is located within the portion spanning from nucleotide 1 to nucleotide 1981 of SEQ ID NO: 13; another preferred target region is

located within the portion spanning from nucleotide 2378 to nucleotide 3252 of SEQ ID NO: 13.

For Erum 8, a preferred target region is located within the portion spanning from nucleotide 1 to nucleotide 926 of SEQ ID NO: 15; another preferred target region is located within the portion spanning from nucleotide 1816 to nucleotide 3570 of SEQ ID NO: 15.

For Erum 9, a preferred target region is located within the portion spanning from nucleotide 1 to nucleotide 1307 of SEQ ID NO: 14; another preferred target region is located within the portion spanning from nucleotide 151 to nucleotide 1836 of SEQ ID NO: 14.

For Erum 10, a preferred target region is located within the portion spanning from nucleotide 1 to nucleotide 598 of SEQ ID NO: 8; another preferred target region is located within the portion spanning from nucleotide 792 to nucleotide 3522 of SEQ ID NO: 8; still another target region is located within the portion spanning from nucleotide 599 to nucleotide 791 of SEQ ID NO: 8.

Various techniques for detection of target nucleic acid sequences based on PCR amplification are available in the art.

These methods include in particular combined PCR analysis, i.e. simultaneous gel visualization of ten individual PCR reactions, each one targeting only one of the genes Erum1 to Erum10 defined above. The ten target genes can also be analysed by multiplex PCR, by a single PCR reaction involving simultaneous amplification of all the genes using a mixture of primers and visualization of the pattern on electrophoresis gel, or by a combination of multiplex PCR reactions, each one concerning a subset of the target genes listed above.

Non-limitative examples of PCR primers allowing to carry out the method of the invention are given in Table 2 below. Other suitable PCR primers can easily be designed by one of skill in the art, on the basis of the information provided by the present invention. By way of non-limitative example of oligonucleotide design software suitable for obtaining PCR primers of the invention, one can mention the software Vector NTI Advance 9.0 (Invitrogene).

The invention also comprises diagnostic kits for discriminating between strains of *E. ruminantium* wherein said kits comprise PCR primers for all the target genes Erum 1 to Erum10.

The method of the invention is useful in particular to discriminate between strains of *E. ruminantium* other than strain Gardel and strain Welgevonden. It is also useful to discriminate between strain Gardel and strains of *E. ruminantium* other than strain Welgevonden, or conversely, between strain Welgevonden and strains of *E. ruminantium*

other than strain Gardel. Furthermore, it also allows for discriminating between a virulent strain of *E. ruminantium* and its attenuated counterpart.

The method of the invention can be performed either on whole bacteria previously lysed, or on nucleic acid (genomic DNA, cDNA or mRNA) isolated from said bacteria. It is suitable for use at various stages of the life cycle of *E. ruminantium*, more specifically but not limited to the domestic-ruminants infectious stage, vector-interaction stage or reservoir animals-interaction stage. Preferred utilisations of the method of the invention include the detection of *Ehrlichia ruminantium* in a given territory, the strain specific identification of *Ehrlichia ruminantium* in a given territory, the discrimination between strains of *Ehrlichia ruminantium* in a given territory or between different geographical regions, the analysis of strain movements within a region or between geographically distinct regions, the differential presence of strains of *Ehrlichia ruminantium* according to vector species and/or populations or the early detection and risk assessment in regions where potential vectors are present but where the disease has not been recorded yet.

Specifically exemplified herein is the identification of *E. ruminantium* strains based on the specific amplification patterns of the ten target genes defined above,

EXAMPLE 1. GENERAL FEATURES AND SEQUENCE REFERENCE

For each strain, purified DNA was broken by sonication to generate fragments of differing sizes. After filling up the ends with Klenow polymerase, DNA fragments ranging from 0.5 kb to 4 kb were separated in a 0.8% agarose gel and collected after gelase (Epicentre) digestion of a cut agarose band. Blunt-end DNA fragments were inserted into pBluescript II KS (Stratagene) digested with EcoRV and dephosphorylated. Ligation was performed with the Fast-Link DNA Ligation kit (Epicentre) and competent DH10B *E. coli* were transformed prior to colony isolation on LB-agar+ Ampicillin + Xgal +IPTG. About 15000 clones were isolated for each strain of *E. ruminantium*. Plasmidic DNA from recombinant *E. coli* strains was extracted according to the alkaline lysis method and inserts were sequenced on both strands using universal forward and reverse M13 primers and the ET DYEnamic terminator kit (Amersham). Sequences were obtained with ABI 373 et ABI 377 automated sequencers (Applied Biosystems). Data were analysed and contigs were assembled using Phred-Phrap and Consed software packages (http://www.genome.washington.edu). Gaps were filled in through primer-directed sequencing using custom made primers. A total of about 20000 raw sequence runs were generated and analysed for each *E. ruminantium* strain to generate a full length consensus sequence with a coverage of 6x to 7x.

E. ruminantium strain Gardel and *E. ruminantium* strain Welgevonden are virulent pathogenic strains causing heartwater in Guadeloupe Island (French West Indies) and South Africa, respectively. The genome of *E. ruminantium* strains Gardel and Welgevonden is arranged as a circular chromosome of 1499920 bp and 1512977 bp, respectively. The

respective G+C contents for the strains Gardel and Welgevonden is 27.51 % and 27.48 %. The genome of *E. ruminantium* strain Gardel comprises 948 coding sequences of an average size of 1018 bp which represent a total coding surface of 63 % of the whole genome. The genome of *E. ruminantium* strain Welgevonden bears 957 genes of the same average size of 1018 bp. The genome surface of this strain devoted to coding sequences is 62 %. Both genomes comprise 36 transfer RNAs (tRNA) and 3 ribosomal RNAs (rRNA).

EXAMPLE 2. IDENTIFICATION OF TARGET GENES FOR STRAIN SPECIFIC DIFFERENTIAL DIAGNOSTIC IN THE GARDEL AND WELGEVONDEN STRAINS OF *E. RUMINANTIUM*

The differential analysis of the whole genomes of *E. ruminantium* strains Gardel and Welgevonden showed the presence of coding sequences which are present in only one of the strains and not in the other. Some of the CDS which are unique to *E. ruminantium* strain Gardel and found only in the genome of this strain are presented in Table 1 (Seq ID NO 1 to Seq ID NO 5). One of the CDS which is unique to *E. ruminantium* strain Welgevonden and found only in the genome of this strain is presented in Table 1 (Seq ID NO 6). Since these sequences are unique to one or the other strain, they clearly represent targets for the differential detection of *E. ruminantium* strain Gardel versus *E. ruminantium* strain Welgevonden.

The differential analysis of the whole genomes of *E. ruminantium* strains Gardel and Welgevonden also showed the presence of coding sequences which are affected by one or several mutations in one of the two strains and for which a non-mutated, functionally active and normal allele is present in the genome of the other strain. Mutations yielded a stop codon which may result in shorter but still predicted CDS depending upon the size of the remaining fragments. Truncated genes resulting in a single CDS are denominated partial CDS, whereas those resulting in two or more predicted CDS are described as fragmented CDS. These coding sequences are presented in Table 1. One Such CDS in the genome of *E. ruminantium* strain Gardel which is affected by mutations and differs from its native counterpart in *E. ruminantium* strain Welgevonden is presented in Table 1 (SEQ ID NO 7). This is a truncated version of the native gene in *E. ruminantium* strain Welgevonden (Table 1, SEQ ID NO 8). The genome of *E. ruminantium* strain Welgevonden also bears mutated genes, with respect to their allelic variant counterparts in the genome of *E. ruminantium* strain Gardel. Three of these CDS which are affected by mutations generating a truncated version of the genes are presented in Table 1 (SEQ ID NO 9 to SEQ ID NO 12). The native full length allele of these CDS present in the genome of *E. ruminantium* strain Gardel are shown in Table 1 (SEQ ID NO 13 to SEQ ID NO 15). One series of CDS in *E. ruminantium* strain Welgevonden (SEQ ID NO 11 and SEQ ID NO 12), whose native full length alleles are found in the genome of *E. ruminantium* strain Gardel (Table 1, SEQ ID NO 15) was affected by mutations generating a frameshift.

Table 1. Target genes for strain-specific differential diagnostic of *E. ruminantium*

Target gene	**Gene in Gardel**	**Status**	**Gene in Welgevonden**	**Status**
Erum 3	ERGA_CDS_05600 (SEQ ID No 1)	Unique gene	None	Sequence absent (full deletion)
Erum 5	ERGA_CDS_07600 (SEQ ID No 2)	Unique gene	None	Sequence absent (full deletion)
Erum 2	ERGA_CDS_04990 (SEQ ID No 3)	Unique gene	None	Partial deletion
Erum 4	ERGA_CDS_05610 (SEQ ID No 4)	Unique gene	None	Extensive mutations
Erum 6	None	Partial deletion	ERWE_CDS_08340 (SEQ ID No 5)	Unique gene
Erum 1	ERGA_CDS_04350 (SEQ ID No 6)	Unique gene	None	Extensive mutations
Erum 10	ERGA_CDS_07340 (SEQ ID No 7)	Partial deletion	ERWE_CDS_07420 (SEQ ID No 8)	Full length gene
Erum 7	ERGA_CDS_01350 (SEQ ID No 13)	Full length gene	ERWE_CDS_01390 (SEQ ID No 9)	Partial deletion
Erum 9	ERGA_CDS_05750 (SEQ ID No 14)	Full length gene	ERWE_CDS_05840 (SEQ ID No 10)	Partial deletion
Erum 8	ERGA_CDS_04510 (SEQ ID No 15)	Full length gene	ERWE_CDS_04590 (SEQ ID No 11) ERWE_CDS_04600 (SEQ ID No 12)	Frameshift (partial deletion)

EXAMPLE 3. DIFFERENTIAL DETECTION OF STRAIN GARDEL AND STRAIN WELGEVONDEN OF *E. RUMINANTIUM* BASED ON PCR AMPLIFICATION PATTERNS OF THE TARGET GENES

Differential PCR identification of strains Gardel and Welgevonden of *E. ruminantium* was achieved using primers described in Table 2.

Table 2

Target gene	Primer name	SEQ ID	Orientation	Size (mer)	CDS
Erum 1	P-Erum 1-A	SEQ ID #16	Sense	21	ERGA_CDS_04350
Erum 1	P- Erum 1-B	SEQ ID #17	Antisense	21	ERGA_CDS_04350
Erum 2	P- Erum 2-A	SEQ ID #18	Sense	25	ERGA_CDS_4990
Erum 2	P- Erum 2-B	SEQ ID #19	Antisense	23	ERGA_CDS_4990
Erum 3	P- Erum 3-A	SEQ ID #20	Sense	20	ERGA_CDS_05600
Erum 3	P- Erum 3-B	SEQ ID #21	Antisense	20	ERGA_CDS_05600
Erum 4	P- Erum 4-A	SEQ ID #22	Sense	19	ERGA_CDS_05610
Erum 4	P- Erum 4-B	SEQ ID #23	Antisense	22	ERGA_CDS_05610
Erum 5	P- Erum 5-A	SEQ ID #24	Sense	23	ERGA_CDS_07600
Erum 5	P- Erum 5-B	SEQ ID #25	Antisense	19	ERGA_CDS_07600
Erum 6	P- Erum 6-A	SEQ ID #26	Sense	26	ERWE_CDS_08340
Erum 6	P- Erum 6-B	SEQ ID #27	Antisense	23	ERWE_CDS_08340
Erum 7	P- Erum 7-A	SEQ ID #28	Sense	25	ERGA_CDS_01350 ERWE_CDS_01390
Erum 7	P- Erum 7-B	SEQ ID #29	Antisense	25	ERGA_CDS_01350 ERWE_CDS_01390
Erum 8	P- Erum 8-A	SEQ ID #30	Sense	25	ERGA_CDS_04510 ERWE_CDS_04590 ERWE_CDS_04600
Erum 8	P- Erum 8-B	SEQ ID #31	Antisense	25	ERGA_CDS_04510 ERWE_CDS_04590 ERWE_CDS_04600
Erum 9	P- Erum 9-A	SEQ ID #32	Sense	25	ERGA_CDS_05750 ERWE_CDS_05840
Erum 9	P- Erum 9-B	SEQ ID #33	Antisense	25	ERGA_CDS_05750 ERWE_CDS_05840
Erum 10	P- Erum 10-A	SEQ ID #34	Sense	25	ERGA_CDS_07340 ERWE_CDS_07420
Erum 10	P- Erum 10-B	SEQ ID #35	Antisense	25	ERGA_CDS_07340 ERWE_CDS_07420

DNA is extracted from elementary bodies of *E. ruminantium,* as described by Perez *et al.* (1997). *E. ruminantium* strains are grown in BUEC cells as described above. Elementary bodies are purified from the culture supernatant by differential centrifugation and resuspended in 350 µl of PBS to which is added 150 µl of buffer containing 25 mM Tris-HCl (pH 8.0), 10 mM MgCl2 and 125 µg of DNase in order to remove contaminating host cell DNA. After incubation for 90 min. at 37°C, the reaction is stopped by addition of 25 mM EDTA. Elementary bodies are washed three times in water and lysed by overnight incubation at 55°C in a solution of 100 mM Tris-HCl (pH 8.0), 150 mM NaCl, 25 mM EDTA, 1.5% SDS and 250 µg/ml of proteinase K. Bacterial DNA is extracted with phenol-choloroform,

precipitated with cold ethanol an resuspended in sterile distilled water. Contamination with cell DNA is evaluate by slot blot hybridization using labeled bovine DNA as a probe and dilutions of bovine DNA (12.5 ng and 25 ng) as positive controls.

PCR amplification of amplicons is performed by mixing 250 ng of *E. ruminantium* DNA, 2.5 U of Taq DNA polymerase, 200 nM of each dNTP, 1 µM of each, sense and antisense, primer and 3 mM $MgCl_2$ in a final volume of 50 µl. Amplification is done under the following conditions: 5 min denaturation at 94°C, followed by 30 cycles of amplification with with a 1-min denaturation, 45 sec of annealing at 45°C and 2 min extension at 72°C. An extra extension step of 10 min at 72°C is added after completion of the 30 cycles. PCR products, i.e. amplicons, are analysed by 1% agarose gel electrophoresis in Tris-borate-EDTA buffer.

The results are summarized in Table 3, Figure 1, and Figure 2.

Legend of Figure 1;

1: Molecular weight marker (100-bp ladder); 2: *Erga* with primers P-Erum 1-A + P-Erum 1-B; 3: *Erwe* with primers P-Erum 1-A + P-Erum 1-B; 4: Control sample with primers P-Erum 1-A + P-Erum 1-B; 5: *Erga* with primers P-Erum 2-A + P-Erum 2-B; 6: *Erwe* with primers P-Erum 2-A + P-Erum 2-B; 7: Control sample with primers P-Erum 2-A + P-Erum 2-B; 8: *Erga* with primers P-Erum 3-A + P-Erum 3-B; 9: *Erwe* with primers P-Erum 3-A + P-Erum 3-B; 10: Control sample with primers P-Erum 3-A + P-Erum 3-B; 11: *Erga* with primers P-Erum 4-A + P-Erum 4-B; 12: *Erwe* with primers P-Erum 4-A + P-Erum 4-B; 13: Control sample with primers P-Erum 4-A + P-Erum 4-B; 14: *Erga* with primers P-Erum 5-A + P-Erum 5-B; 15: *Erwe* with primers P-Erum 5-A + P-Erum 5-B; 16: Control sample with primers P-Erum 5-A + P-Erum 5-B; 17: *Erga* with primers P-Erum 6-A + P-Erum 6-B; 18: *Erwe* with primers P-Erum 6-A + P-Erum 6-B; 19: Control sample with primers P-Erum 6-A + P-Erum 6-B; 20: Molecular weight marker (1 *Eco*RI-*Hin*dIII).

Legend of Figure 2:

1: Molecular weight marker (100-bp ladder); 2: *Erga* with primers P-Erum 7-A + P-Erum 7-B; 3: *Erwe* with primers P-Erum 7-A + P-Erum 7-B; 4: Control sample with primers P-Erum 7-A + P-Erum 7-B; 5: *Erga* with primers P-Erum 9-A + P-Erum 9-B; 6: *Erwe* with primers P-Erum 9-A + P-Erum 9-B; 7: Control sample with primers P-Erum 9-A + P-Erum 9-B; 8: *Erga* with primers P-Erum 10-A + P-Erum 10-B; 9: *Erwe* with primers P-Erum 10-A + P-Erum 10-B; 10: Control sample with primers P-Erum 10-A + P-Erum 10-B; 11: Molecular weight marker (1 *Eco*RI-*Hin*dIII); 12: Molecular weight marker (100-bp ladder); 13: *Erga* with primers P-Erum 8-A + P-Erum 8-B; 14: *Erwe* with primers P-Erum 8-A + P-Erum 8-B; 15: Control sample with primers P-Erum 8-A + P-Erum 8-B

As shown in Table 3, Fig.1 and Fig. 2, all the pairs of primers described in Table 3 allowed for differential indentification and discrimination of strains Gardel and Welgevonden. The PCR reactions yielded the results expected from *in silico* prediction of

amplicons (Table 3). The pairs P-Erum 1-A + P-Erum 1-B, P-Erum 2-A + P-Erum 2-B, P-Erum 3-A + P-Erum 3-B, P-Erum 4-A + P-Erum 4-B, P-Erum 5-A + P-Erum 5-B, which target unique genes present only in strain Gardel generated the expected unique amplicons of 172 bp, 217 bp, 508 bp, 642 bp and 238 bp, respectively, on strain Gardel while generating no bands on strain Welgevonden (Table 3, Fig. 1). The pair of primers P-Erum 6-A + P-Erum 6-B which target a unique gene only present in strain Welgevonden yielded, as expected, a single amplicon of 127 bp while no PCR product was obtained on strain Gardel (Table 3, Fig. 1). The pairs P-Erum 7-A + P-Erum 7-B, P-Erum 8-A + P-Erum 8-B, P-Erum 9-A + P-Erum 7-B and P-Erum 10-A + P-Erum 10-B targeting the truncated genes yielded PCR products of the respective expected size of 2791 bp, 552 bp+ 1071 bp, 1361 bp and 1095 bp on strain Gardel and 2395 bp, 492 bp, 1178 bp and 1691 bp on strain Welgevonden, respectively (Table 3, Fig. 2). An additional band of 480 bp is observed on strain Gardel with the pair P-Erum 8-A + P-Erum 8-B. This additional band is most likely due to a single low specificity response occurring in *Erga*.

The primer pairs P-1350-A + P-1350-B, P-4510-A + P-4510-B, P-5750-A + P-5750-B and P-7420-A + P-7420-B yielded PCR products of the respective expected size of 2791, 552+1071, 1361 and 1095 on strain Gardel and 2395, 492, 1178 and 1691 on strain Welgevonden, respectively (Table 3, Fig. 1 and Fig. 2). An additional band of 480 bp is observed on strain Gardel with the pair P-4510-A + P-4510-B. This additional band is most likely due to a single low specificity response occurring in strain Gardel.

Table 3. Strain-specific differential PCR screening of *E. ruminantium* strain Gardel and strain Welgevonden

Primer combination	Strain			
	Gardel		Welgevonden	
	Expected	Observed	Expected	Observed
P-Erum 1-A + P-Erum 1-B	172	172	None	None
P-Erum 2-A + P-Erum 2-B	217	217	None	None
P-Erum 3-A + P-Erum 3-B	508	508	None	None
P-Erum 4-A + P-Erum 4-B	642	642	None	None
P-Erum 5-A + P-Erum 5-B	238	238	None	None
P-Erum 6-A + P-Erum 6-B	None	None	127	127
P-Erum 7-A + P-Erum 7-B	2791	2791	2395	2395
P-Erum 8-A + P-Erum 8-B	552+1071	552+1071+ 480	492	492
P-Erum 9-A + P-Erum 9-B	1361	1361	1178	1178
P-Erum 10-A + P-Erum 10-B	1095	1095+300	1691	1691

EXAMPLE 4: DIFFERENTIAL STRAIN-SPECIFIC PCR DETECTION AND IDENTIFICATION OF STRAINS OF *E. RUMINANTIUM* DIFFERENT THAN STRAIN GARDEL AND STRAIN WELGEVONDEN

The use primers listed in Table 2 were used for the specific identification and discrimination of *E .ruminantium* strains other than strain Gardel and strain Welgevonden. The strains others than Gardel and Welgevonden presented in this example are strains Umpala (Mozambique), Senegal (Senegal), Bankouma (Burkina Faso) , Bekuy (Burkina Faso), Lamba (Burkina Faso), Banan 1 (Burkina Faso) and Banan 2 (Burkina Faso).

These strains are presented here to illustrate samples from different parts of Sub-Saharan Africa and the Caribbean.

DNA is extracted from elementary bodies of *E. ruminantium* and PCR amplification performed as described in Example 3.

The results are shown in Table 4, Fig. 3 and Fig. 4:

Legend of Figure 3:

A. PCR detection with primers P-Erum 1-A + P-Erum 1-B
B. PCR detection with primers P-Erum 3-A + P-Erum 3-B
C. PCR detection with primers P-Erum 2-A + P-Erum 2-B
D. PCR detection with primers P-Erum 4-A + P-Erum 4-B
E. PCR detection with primers P-Erum 6-A + P-Erum 6-B
F. PCR detection with primers P-Erum 5-A + P-Erum 5-B

MW1: Molecular weight marker (100pb DNA ladder); MW2: Molecular weight marker (l *Hin*dIII/*Eco*RI); 1: Strain Senegal attenuated (Satt); 2: Strain Gardel CTVM; 3: Strain Bankouma; 4: Strain Bekuy; 5: Strain Lamba, 6: Strain Banan 1; 7: Strain Banan 2; NC: Negative control; G. Strain Gardel; W: Strain Welgevonden.

Legend of Figure 4:

A. PCR detection with primers P-Erum 7-A + P-Erum 7-B
B. PCR detection with primers P-Erum 8-A + P-Erum 8-B
C. PCR detection with primers P-Erum 9-A + P-Erum 9-B
D. PCR detection with primers P-Erum 10-A + P-Erum 10-B

MW1: Molecular weight marker (100pb DNA ladder); MW2: Molecular weight marker (l *Hin*dIII/*Eco*RI); 1: Strain Bankouma; 2: Strain Bekuy; 3: Strain Lamba; 4: Strain Banan 1; 5: Strain Banan 2; 6: Strain Gardel attenuated (Gatt); 7: Strain Gardel CTVM; 8: Strain Senegal; 9: Strain Senegal attenuated (Satt); NC: Negative control; G. Strain Gardel; W: Strain Welgevonden.

As shown in Table 4, Fig. 3 and Fig. 4, the combined use of all the pairs of primers described in Table 2 allowed for differential identification and discrimination of strains other than strains Gardel and Welgevonden. The PCR reactions results are summarized in Table 4. The pairs P-Erum 1-A + P-Erum 1-B, P-Erum 2-A + P-Erum 2-B, P-Erum 3-A + P-Erum 3-B, P-Erum 4-A + P-Erum 4-B, P-Erum 5-A + P-Erum 5-B which target unique genes present only in strain Gardel and the pair P-Erum 6-A + P-Erum 6-B which targets a unique gene only present in strain Welgevonden all yieleded differing patterns of PCR products depending on the strain (Table 4, Fig 3). Differing patterns depending upon the strain were also observed using the pairs P-Erum 7-A + P-Erum 7-B, P-Erum 8-A + P-Erum 8-B, P-Erum 9-A + P-Erum 7-B and P-Erum 10-A + P-Erum 10-B which target the truncated genes (Table 4, Fig. 3).

It is however the overall analysis of all the PCR patterns yielded by all the pairs of primers described in Table 2 which provides a strain specific diagnostic. The strains Bekuy and Lamba which were isolated in Burkina Faso from the nearby villages of Bekuy and Lamba, respectively, are most likely to be two isolates of the same strain. Furthermore, these strains display the same map-1 genotype determined by PCR amplification and sequencing of the map-1 gene. All the other strains display differing map-1 genotypes. This further indicates that strains Bekuy and Lamba are two isolate of the same strain. The identical overall pattern obtained for these two strains with all the pairs of primers described in Table 2 also further demonstrate the strain-specificity of the subject of the invention and its ability to identify different strains and separate isolates of the same strain.

Table 4: Strain-specific differential PCR screening of *E. ruminantium*

Primer combination	Strain								
	Gardel	**Welgevonden**	**Umpala**	**Senegal**	**Bankouma**	**Bekuy**	**Lamba**	**Banan1**	**Banan2**
P-Erum 1-A + P-Erum 1-B	172	None	172	172	Multibands	172	172	172	Multibands
P-Erum 2-A + P-Erum 2-B	217	None	515	500 + 900	280+500+1200	500+900	500+900	500+900	500+1200
P-Erum 3-A + P-Erum 3-B	508	None	508	None	560	508+1900	508+1900	None	508
P-Erum 4-A + P-Erum 4-B	642	None	642	642	642 [a]	642	642	642	642
P-Erum 5-A + P-Erum 5-B	238	None	238	None	238	238	238	238	238
P-Erum 6-A + P-Erum 6-B	None	127	None	127	None	127	127	127	None
P-Erum 7-A + P-Erum 7-B	2791	2395	2791+ 500	2395	2395	2395	2395	2395	None
P-Erum 8-A + P-Erum 8-B	552+1071 +480	492	1200 + 500[a]	492	492	492	492	492	552+1071 +480
P-Erum 9-A + P-Erum 9-B	1361	1178	1361	1000	1000	1000	1000	1000	1178+1361
P-Erum 10-A + P-Erum 10-B	1095+300	1691	820	820	820	820	820	820	1095

a: presence of additional multiple bands is observed

EXAMPLE 5: DIFFERENTIAL SPECIFIC PCR DETECTION AND IDENTIFICATION OF ATTENUATED AND DIFFERING DERIVATES OF STRAINS GARDEL AND SENEGAL

The primers listed in Table 2 also allow the specific identification of attenuated variants of known strains of *E. ruminantium.*

The following variants were tested:

- attenuated derivates of strains Gardel and Senegal denominated Gatt (for Gardel-attenuated) and Satt (for Senegal-attenuated), respectively. The strain Gatt was obtained from the virulent strain Gardel through 248 successive passages BUEC cells whereas strain Satt was obtained from the virulent strain Senegal following 64 passages on BUEC cells. Both the Gatt and Satt strains display an attenuated phenotype characterized by a lack of virulence.

- strain Gardel CTVM, which is a subset of strain Gardel maintained in a differing cell environment, and was reported has having undergone mutations in the *map1* operon and displaying a diverging phenotype (Bekker *et al.*, 2004).

DNA is extracted from elementary bodies of *E. ruminantium* and PCR amplification performed as described in Example 3.

The results are shown in Table 5, Figure 4, and Figure 5.

Legend of Figure 4:

A. PCR detection with primers P-Erum 7-A + P-Erum 7-B

B. PCR detection with primers P-Erum 8-A + P-Erum 8-B

C. PCR detection with primers P-Erum 9-A + P-Erum 9-B

D. PCR detection with primers P-Erum 10-A + P-Erum 10-B

MW1: Molecular weight marker (100pb DNA ladder); MW2: Molecular weight marker (l *Hin*dIII/*Eco*RI); 1: Strain Bankouma; 2: Strain Bekuy; 3: Strain Lamba; 4: Strain Banan 1; 5: Strain Banan 2; 6: Strain Gardel attenuated (Gatt); 7: Strain Gardel CTVM; 8: Strain Senegal; 9: Strain Senegal attenuated (Satt); NC: Negative control; G. Strain Gardel; W: Strain Welgevonden.

Legend of Figure 5:

- A. PCR analysis of virulent and attenuated strains with primers P-Erum 1-A + P-Erum 1-B

MW1: Molecular weight marker (100-pb DNA ladder); MW2: Molecular weight marker (l *Hin*dIII/*Eco*RI); 1: Strain Gardel attenuated (Gatt); 2: Strain Gardel; 3: Negative control.

- B. PCR analysis of virulent and attenuated strains with primers P-Erum 2-A + P-Erum 2-B and P-Erum 6-A + P-Erum 6-B

MW1: Molecular weight marker (100-pb DNA ladder); MW2: Molecular weight marker (l *Hin*dIII/*Eco*RI); Analysis with P-Erum 2-A + P-Erum 2-B of 1: Strain Gardel attenuated (Gatt); 2: Strain Gardel; 3: Negative control; Analysis with P-Erum 6-A + P-Erum 6-B of 4:

Strain Gardel attenuated (Gatt); 5: Strain Gardel; 6: Strain Welgevonden; 3: Negative control.

- C. PCR analysis of virulent and attenuated strains with primers P-Erum 3-A + P-Erum 3-B, P-Erum 4-A + P-Erum 4-B and P-Erum 5-A + P-Erum 5-B

MW1: Molecular weight marker (100-pb DNA ladder); MW2: Molecular weight marker (l *Hin*dIII/*Eco*RI); Analysis with P-Erum 4-A + P-Erum 4-B of 1: Strain Gardel attenuated (Gatt); 2: Strain Gardel; 3: Negative control; Analysis with P-Erum 5-A + P-Erum 5-B of 4: Strain Gardel attenuated (Gatt); 5: Strain Gardel; 6: Negative control; Analysis with P-Erum 3-A + P-Erum 3-B of 7: Strain Gardel attenuated (Gatt); 8: Strain Gardel; 9: Strain Gardel; 10: Negative control.

As shown in Table 5, Fig. 4 and Fig. 5, the combined use of all the pairs of primers described in Table 2 also allowed for the differential identification and discrimination of variants and attenuated derivates of known strains. The PCR reactions results are summarized in Table 5. The overall PCR patterns generated on the strain Gardel and and two of its derivates, the strain Gardel CTVM and the attenuated strain Gatt show the presence of slight variations –Table 5, Fig. 4, Fig. 5). Furthermore, each strain is characterized by a specific pattern. The strain Gatt differs from the parental virulent strain Gardel by the products from primers pairs P-Erum 6-A + P-Erum 6-B and P-Erum 7-A + P-Erum 7-B, whereas the strain Gardel CTVM differs from the parental strain Gardel by the product from the primers pair P-Erum 6-A + P-Erum 6-B. Similarly, the primers pair P-Erum 7-A + P-Erum 7-B allows for discrimination between the strain Gardel CTVM and the attenuated Gardel strain Gatt. A similar situation is observed between the parental virulent strain Senegal and its attenuated derivate Satt (Table 5, Fig. 4). The virulent strain Senegal and the attenuated strains Satt differ by the PCR product from the primer pairs P-Erum 2-A + P-Erum 2-B, P-Erum 3-A + P-Erum 3-B, P-Erum 6-A + P-Erum 6-B and P-Erum 7-A + P-Erum 7-B.

Table 5: Differential identification of attenuated Gardel and Senegal strains of *E. ruminantium*

Primer combination	Strain				
	Gardel (Virulent)	Gatt (attenuated Gardel)	Gardel CTVM	Senegal	Satt (attenuated Senegal)
P-Erum 1-A + P-Erum 1-B	172	172	172	172	172
P-Erum 2-A + P-Erum 2-B	217	217	217	500 + 900	500 + 900+210
P-Erum 3-A + P-Erum 3-B	508	508	508	None	508+1900
P-Erum 4-A + P-Erum 4-B	642	642	642	642	642
P-Erum 5-A + P-Erum 5-B	238	238	238	None	238
P-Erum 6-A + P-Erum 6-B	None	127	127	127	127
P-Erum 7-A + P-Erum 7-B	2791	None	2791	2395	2395+700+300
P-Erum 8-A + P-Erum 8-B	552+1071+ 480	552+1071+ 480	552+1071+ 480	492	492
P-Erum 9-A + P-Erum 9-B	1361	1361	1361	1000	1000
P-Erum 10-A + P-Erum 10-B	1095+300	1095+300	1095+300	820	820

EXAMPLE 6: ABSENCE OF CROSS-REACTION WITH OTHER RICKETTSIALES

To verify the specificity assessment of the primers listed in Table 2, they were tested on *Rickettsiales* belonging to other species and genera than *E. ruminantium* i.e. *Ehrlichia canis, Anaplasma platys* and *Anaplasma marginale.*

DNA extraction and PCR amplification were performed as described in Example 3.

The results are shown in Figures 6, 7, and 8.

Legend of Figure 6:

- A. PCR detection with probe EHR16S specific to *Ehrlichia spp.* 16S rDNA
MW: Molecular weight marker (100-bp ladder); 1, 2 and 3: DNA isolated from blood samples from dogs infected with *Anaplasma platys*, 4, 5 and 8: DNA isolated from blood samples from dogs infected with *Ehrlichia canis;* 6: *Ehrlichia canis* positive control; 7: Negative control; 9 and 10: Control DNA isolated from blood samples from non-infected dogs.
- B. PCR detection with Nested PCR probes specific to *Anaplasma platys*
MW: Molecular weight marker (100-bp ladder); 1, 2 and 3: DNA isolated from blood samples from dogs infected with *Anaplasma platys.*

- C. PCR detection with Nested PCR probes specific to *Ehrlichia canis*

MW: Molecular weight marker (100-bp ladder); 4, 5and 8: DNA isolated from blood samples from dogs infected with *Ehrlichia canis;* 11: DNA from canine monocytes cultures infected with *E. canis* (supernatant); 12: DNA from canine monocytes cultures infected with *E. canis* (pellet).

Legend of Figure 7:

A. PCR detection with primers P-Erum 1-A + P-Erum 1-B
B. PCR detection with primers P-Erum 2-A + P-Erum 2-B
C. PCR detection with primers P-Erum 3-A + P-Erum 3-B
D. PCR detection with primers P-Erum 4-A + P-Erum 4-B
E. PCR detection with primers P-Erum 5-A + P-Erum 5-B
F. PCR detection with primers P-Erum 6-A + P-Erum 6-B

MW1: Molecular weight marker (100pb DNA ladder); MW2: Molecular weight marker (l *Hin*dIII/*Eco*RI); 1, 2 and 3: DNA isolated from blood samples from dogs infected with *Anaplasma platys*, 4, 5 and 8: DNA isolated from blood samples from dogs infected with *Ehrlichia canis;* 9 and 10: Control DNA isolated from blood samples from non-infected dogs, 11: DNA from canine monocytes cultures infected with *E. canis (*supernatant); 12: DNA from canine monocytes cultures infected with *E. canis* (pellet); Am: DNA from *Anaplasma marginale*; G: DNA from strain Gardel; NC: Negative control.

Legend of Figure 8:

A. PCR detection with primers P-Erum 7-A + P-Erum 7-B
B. PCR detection with primers P-Erum 8-A + P-Erum 8-B
C. PCR detection with primers P-Erum 9-A + P-Erum 9-B
D. PCR detection with primers P-Erum 10-A + P-Erum 10-B

MW1: Molecular weight marker (100pb DNA ladder); MW2: Molecular weight marker (l *Hin*dIII/*Eco*RI); 1, 2 and 3: DNA isolated from blood samples from dogs infected with *Anaplasma platys*, 4, 5 and 8: DNA isolated from blood samples from dogs infected with *Ehrlichia canis;* 9 and 10: Control DNA isolated from blood samples from non-infected dogs, 11: DNA from canine monocytes cultures infected with *E. canis (*supernatant); 12: DNA from canine monocytes cultures infected with *E. canis* (pellet); Am: DNA from *Anaplasma marginale*; G: DNA from strain Gardel; W: DNA from strain Welgevonden; NC: Negative control.

Fig. 6 indicates that the samples used indeed contain DNA from *A. platys* and *E. canis* as demonstrated by their recognition by 16S rDNA-specific primers and primers for nested PCR specific to each species. As shown in Fig. 7 and Fig. 8, the pairs of primers described in Table 2 are strictly specific to *E. ruminantium* and display no cross-reaction with other related *Rickettsiales* since no specific PCR product could be detected on *E. canis, A. platys* and *A. marginale* (Fig. 7 and Fig.8). Whereas no PCR products are detectable on

whatever pair of primers was used, PCR products were visible on *A. platys* and *E. canis*. However, all these PCR products are generated by cross-reactions with canine blood cells as shown by the detection of these same bands on non-infected canine cells (Fig. 7 and Fig. 8). This demonstrate that the primers described in Table 2 and targeting the target genes described in Table 1 allow for specific identification of *E. ruminantium* and discrimination between strains of *E. ruminantium* even when other *Rickettsiales* are present.

The tools provided by the invention allow thus both for specific detection of *E. ruminantium*, even in presence of contaminating related *Ricketssiales*, for specific discrimination between different strains of *E. ruminantium* and for specific discrimination between a virulent strains and its vaccinal attenuated derivates. This in turn allows for monitoring of vaccination.

CLAIMS

1) Use of the following set of target genes :

Erum1, defined by the sequence SEQ ID NO: 6
Erum2, defined by the sequence SEQ ID NO: 3
Erum3, defined by the sequence SEQ ID NO: 1
Erum4, defined by the sequence SEQ ID NO: 4
Erum5, defined by the sequence SEQ ID NO: 2
Erum6, defined by the sequence SEQ ID NO: 5
Erum7, defined by the sequence SEQ ID NO: 13
Erum8, defined by the sequence SEQ ID NO: 15
Erum9, defined by the sequence SEQ ID NO: 14
Erum10, defined by the sequence SEQ ID NO: 8,

for the strain-specific detection of *Ehrlichia ruminantium.*

2) A method for the strain-specific detection of *Ehrlichia ruminantium* wherein said method comprises detecting, for each of the genes Erum1 to Erum10, whether an allele of said gene is present in the bacteria to be tested, and determining the form of said allele.

3) A method of claim 2, which comprises performing PCR amplification of all the target genes Erum 1 to Erum10, and checking, for each of these genes, the presence of one or more amplification product(s), and the size of said amplification product(s).

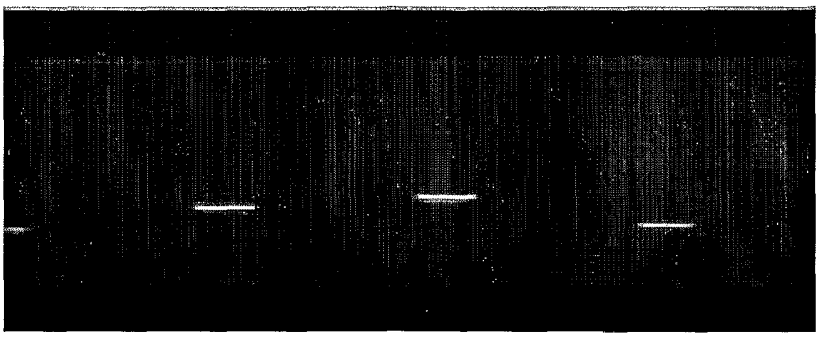

Figure 1

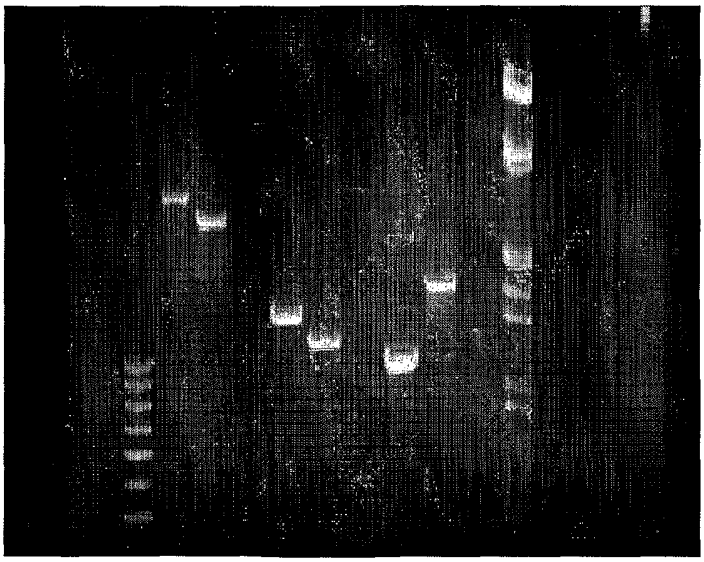

Figure 2

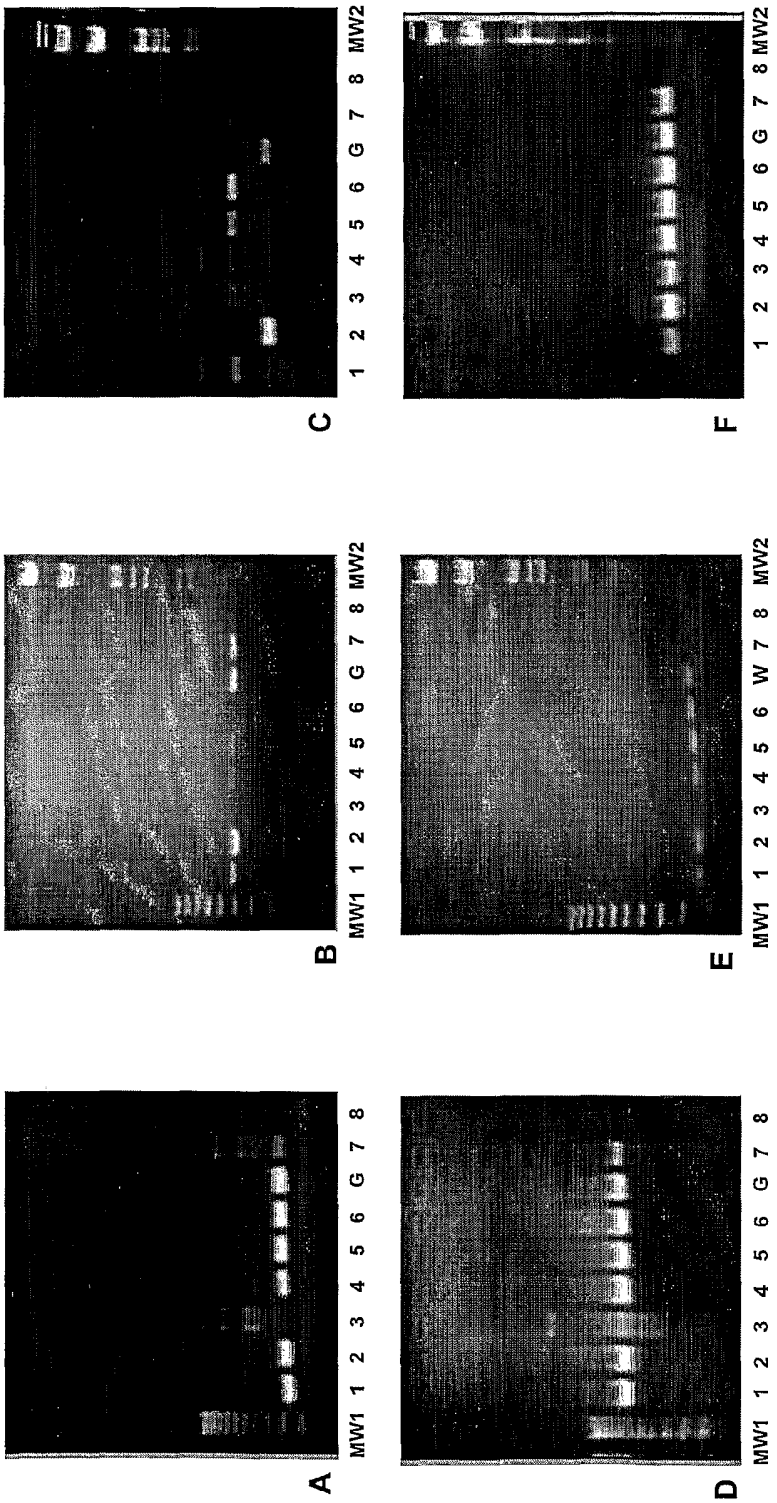
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Figure 3

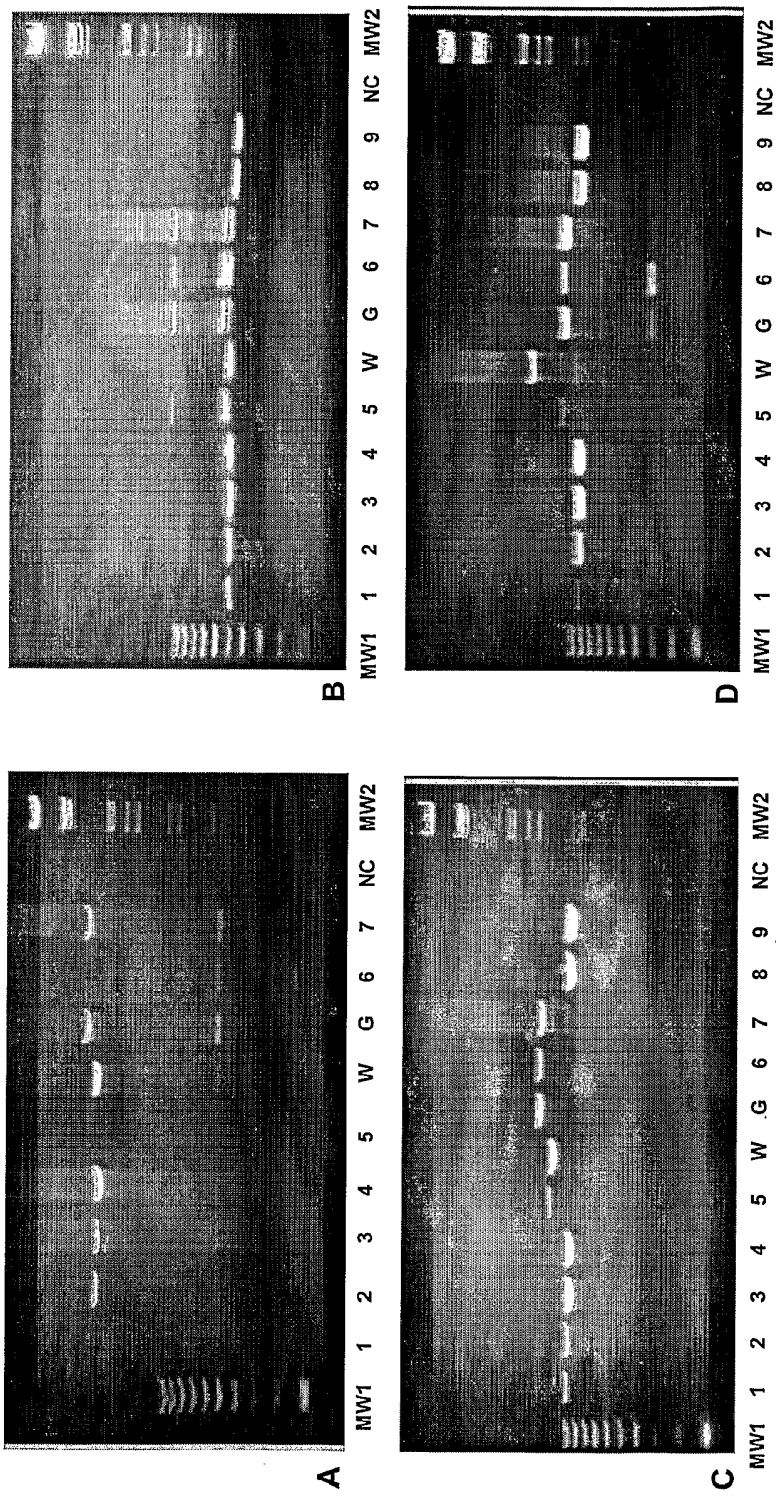
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Figure 4

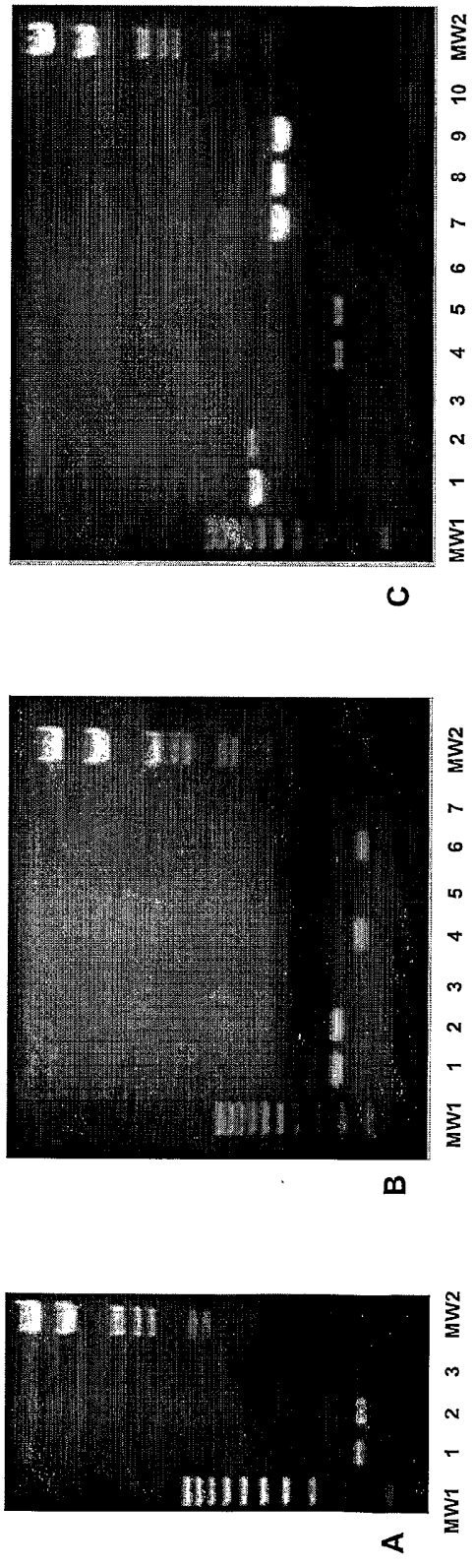


Figure 5

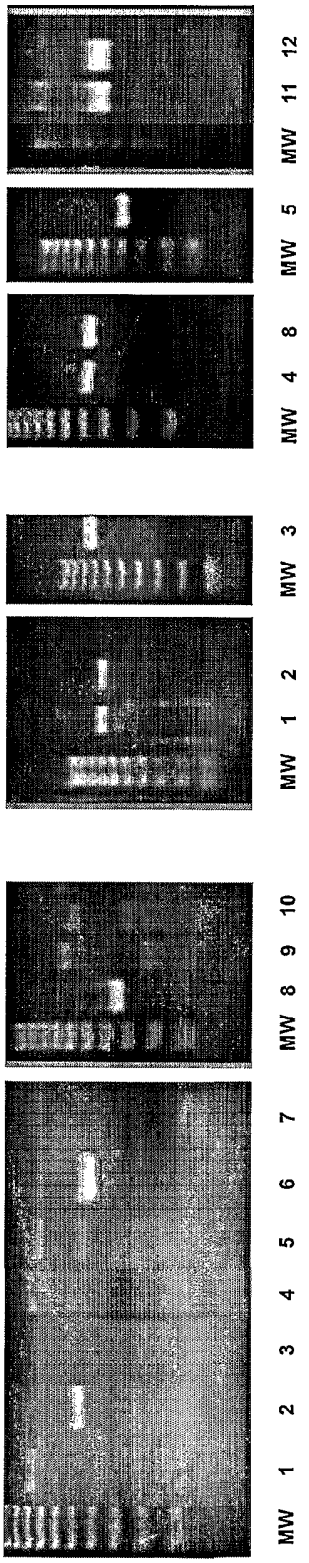
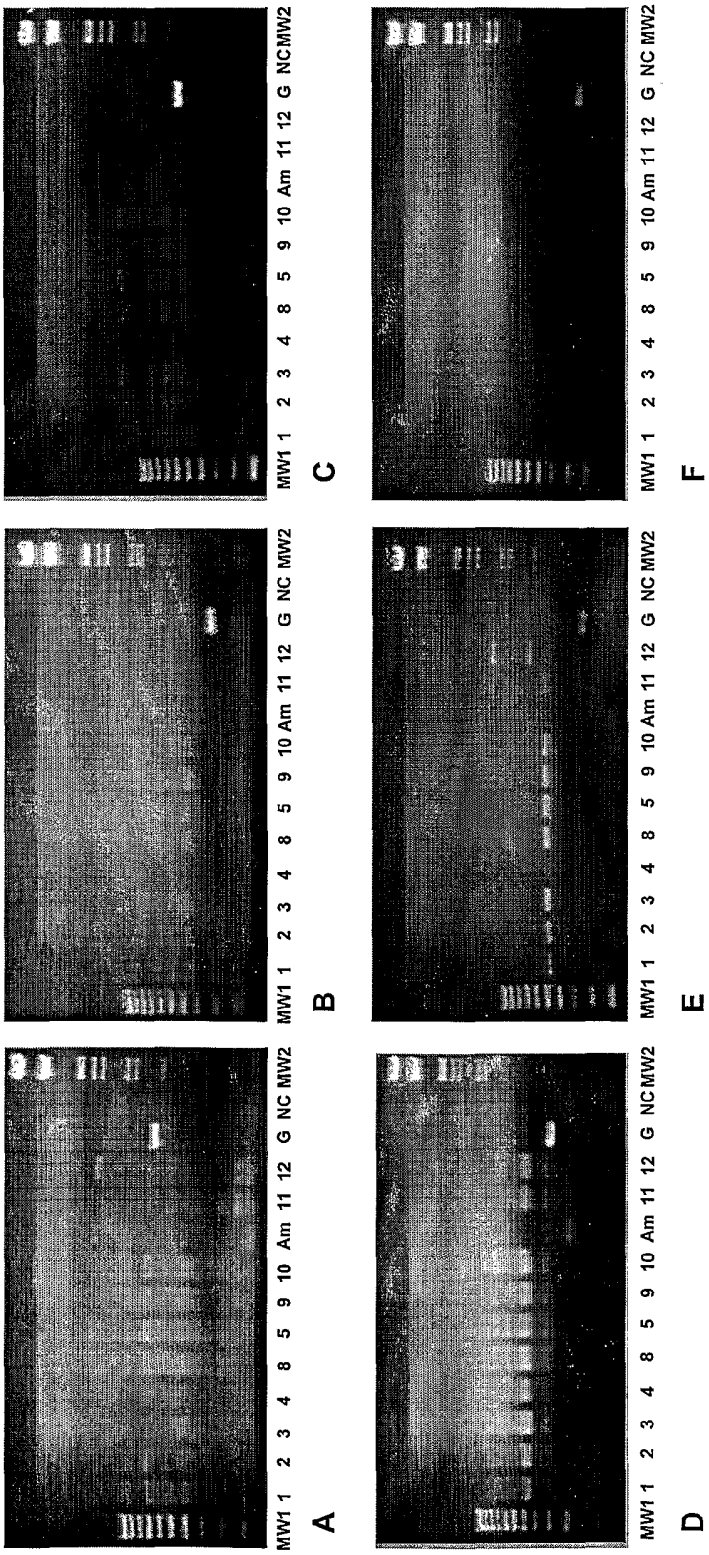


Figure 6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C
MW1 1 2 3 4 8 5 9 10 Am 11 12 G NC MW2
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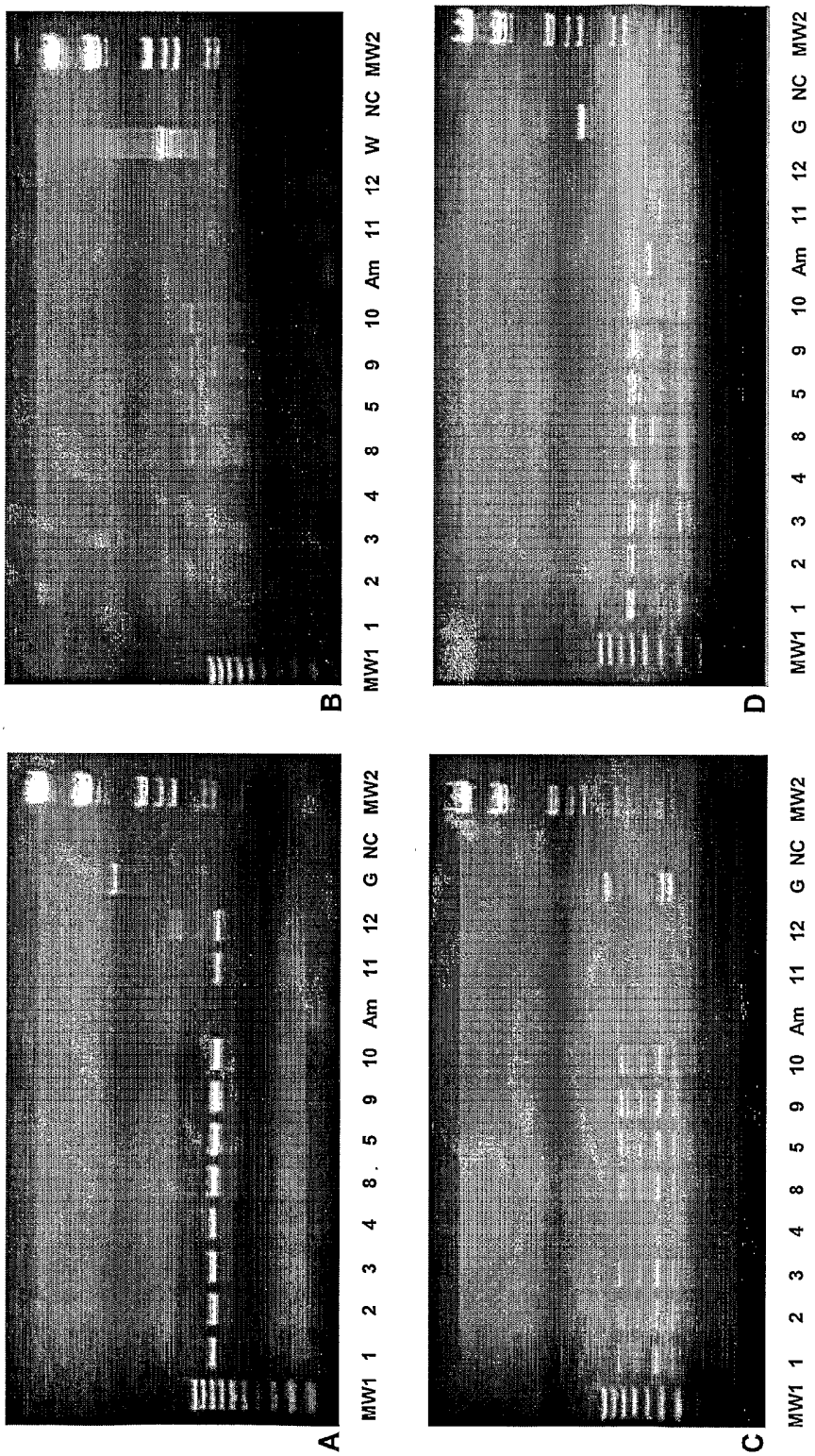
F

Figure 7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B
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Figure 8

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- LEFRANCOIS, Thierry
- FERRAZ, Conception
- DEMAILLE, Jacques
- MARTINEZ, Dominique

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INTERNATIONAL SEARCH REPORT

International application No
PCT/IB2006/003870

A. CLASSIFICATION OF SUBJECT MATTER
INV. C12N1/20 C12N15/09 C12Q1/68

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal, EMBASE, BIOSIS, WPI Data, PAJ

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO 2006/045338 A (AGRONOMIQUE POUR LE DEV CIRAD [FR]; CENTRE NAT RECH SCIENT [FR]; FRUTO) 4 May 2006 (2006-05-04) cited in the application page 2, line 19 - page 14, line 28; sequences 1-6, 9, 11, 13, 20	1-3
A	FRUTOS R ET AL: "Comparative genomic analysis of three strains of Ehrlichia ruminantium reveals an active process of genome size plasticity" JOURNAL OF BACTERIOLOGY 2006 UNITED STATES, vol. 188, no. 7, April 2006 (2006-04), pages 2533-2542, XP002430891 ISSN: 0021-9193 cited in the application the whole document	1-3

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[X] Further documents are listed in the continuation of Box C.

[X] See patent family annex.

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24 April 2007	09/05/2007

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL – 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Donath, Cornelia

INTERNATIONAL SEARCH REPORT

International application No
PCT/IB2006/003870

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	BEKKER CORNELIS P J ET AL: "Transcription analysis of the major antigenic protein 1 multigene family of three in vitro-cultured Ehrlichia ruminantium isolates" JOURNAL OF BACTERIOLOGY, vol. 187, no. 14, July 2005 (2005-07), pages 4782-4791, XP002430892 ISSN: 0021-9193 the whole document	1-3
A	COLLINS NICOLA E ET AL: "The genome of the heartwater agent Ehrlichia ruminantium contains multiple tandem repeats of actively variable copy number" PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF THE UNITED STATES OF AMERICA, vol. 102, no. 3, 18 January 2005 (2005-01-18), pages 838-843, XP002430893 ISSN: 0027-8424 the whole document	1-3
A	ALLSOPP M T ET AL: "Ehrlichia ruminantium major antigenic protein gene (map1) variants are not geographically constrained and show no evidence of having evolved under positive selection pressure" JOURNAL OF CLINICAL MICROBIOLOGY, WASHINGTON, DC, US, vol. 39, no. 11, November 2001 (2001-11), pages 4200-4203, XP002321870 ISSN: 0095-1137 the whole document	1-3

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/IB2006/003870

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 2006045338 A	04-05-2006	NONE	